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Different susceptibility of European grapevine cultivars for downy mildew

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Summary

Downy mildew, caused by the obligately biotrophic peronosporomycete *Plasmopara viticola*, is one of the most destructive of grapevine diseases that occurs worldwide. The classical cultivars of *Vitis vinifera*, up to date utmost important for wine and table grape production, are all susceptible to *P. viticola*, resulting in severe epidemics under warm and humid conditions. The aim of our present study was to characterize the susceptibility to infection by *P. viticola* among different grapevine cultivars grown in European vineyards in comparison to resistant *Vitis* species. For this purpose we inoculated leaf discs, leaves and whole plants of eight *V. vinifera* cultivars considered to be susceptible ('Albariño' [Clone1, Clone2 and Clone3], 'Tempranillo', 'Touriga Nacional', 'Riesling', 'Pinot Noir', 'Pinot Blanc', 'Müller-Thurgau' and 'Cabernet Sauvignon') with *P. viticola* under controlled conditions. Four *Vitis* genotypes with a distinct degree of resistance to *P. viticola* (*V. riparia*, *V. rupestris*, *V. amurensis* and the hybrid *Vitis* x *vinifera* 'Solaris') were used as resistant and partially resistant references. To assess the degree of susceptibility we scored the disease incidence and severity visually and microscopically analyzed the course of host tissue colonization by the pathogen. The microscopical studies indicated even slight differences in the infection rate, the course of host tissue colonization and the parasitation i.e. haustoria formation, among the *V. vinifera* cultivars. The obtained data were suitable for statistical analysis that showed significant differences in the assessed parameters among the *V. vinifera* cultivars. The principal component analysis (PCA) of the data revealed three groups of susceptibility: (i) genotypes which are little susceptible, e.g. 'Cabernet Sauvignon', 'Pinot Blanc', 'Pinot Noir', 'Müller-Thurgau' and 'Riesling'; (ii) a second group formed by those genotypes which are very susceptible, *i.e.* the two clones of 'Albariño' (the most susceptible of all) and 'Tempranillo'; and (iii) a third group comprising the genotypes used as resistant and partially resistant references (*V. riparia*, *V. rupestris*, *V. amurensis* and the hybrid *Vitis* x *vinifera* 'Solaris'). Within the first group 'Cabernet Sauvignon' formed a subgroup indicating a very low susceptibility to *P. viticola*. In this work, for the first time the visual assessment of disease incidence and severity with a microscopical analysis of infection intensity, colonization of host tissue and parasitation to discriminate differences in susceptibility of European *V. vinifera* cultivars for *P. viticola* was combined.

K e y w o r d s : *Plasmopara viticola, Vitis vinifera*, European cultivars, *Vitis* spp., susceptibility, resistance, disease severity, disease incidence.

Introduction

Downy mildew, caused by the obligately biotrophic peronosporomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni., is among of the most destructive grapevine diseases, that occurs worldwide, particularly in warm and humid climates. The pathogen is adapted to the family *Vitaceae*, especially to the subgenus *Euvitis* and endemic to south-eastern North America. *P. viticola* has been collected the first time by Schweinitz (1838) on wild *Vitis* species in South Carolina (Farlow 1883). Most of the *Vitis* taxa endemic in North America are more or less resistant to *P. viticola* probably due to the coevolution between *Vitis* and *P. viticola*. The pathogen has been introduced to Europe in the last quarter of the 19th century and in 1878 the first symptoms were observed in the Bordeaux area. In the following decade all classical European grapevine cultivars showed to be highly susceptible, resulting in a severe pandemic throughout the continent (Millardet 1883, Viala 1887, Ravaz 1911).

A number of authors (Ravaz 1914, Boubals 1959, Ribereau-Gayón and Peinaud 1971, Galet 1995, Staudt and Kassemeyer 1995) indicate, that some taxa of the genus *Vitis* show a wide range of resistance towards downy mildew. *Vitis candicans, V. cinerea, V. cordifolia, V. monticola, V. riparia*, *V. rotundifolia,* and *V. titania* are recorded as highly resistant, *V. lincecumii* and *V. vulpina* as partially resistant, *V. aestivalis, V. arizonica, V. berlandieri, V. doniana, V. palmate,* and *V. rupestris* as partially susceptible, while the European cultivars of *V. vinifera* are generally regarded as highly susceptible (Boubals 1959, Galet 1977, Li 1985, Staudt and Kassemeyer 1995, Staudt 1997; Wiedemann-Merdinoglu *et al*. 2006). Boubals (1959) established a seven point scale ranging from low to high susceptibility. Meluc (1981), Li (1985), and Galet (1995) classified grapevine cultivars into different groups according to their susceptibility to infection. Hybrids between *V. vinifera* and American species, including their multiple backcrossings with European cultivars, often regarded as *Vitis vinifera* cultivars, show a range of resistance (Becker 1994, Basler

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et al. 2002) against *P. viticola*. Several authors (Staudt and Kassemeyer 1995, Spring *et al.* 1998, Brown *et al.* 1999 a, b, Kortekamp and Zyprian 2003, Spring 2003) have studied the resistance of different *Vitis* genotypes against *Plasmopara viticola*. Unger *et al*. (2007) described the temporal and spatial course of host mesophyll colonization by *P. viticola* in a susceptible and a resistant *Vitis* genotype. They described the steps of pathogen development during the colonization of both the susceptible *V. vinifera* 'Müller-Thurgau' and the resistant *V. rupestris*, and found a delayed development of *P. viticola* in the resistant genotype.

Many stages of the infection process of downy mildew are well understood, including the encystation of the zoospores, the germination of spores and the formation of the penetration peg, which invades the host through the stomata (Müller and Sleumer 1934, Kiefer *et al.* 2002, Riemann *et al*. 2002). The duration of the incubation period and the influence of temperature, relative humidity, and leaf wetness on infection, incubation period, and sporulation has been described by Müller and Sleumer (1934) and Blaeser and Weltzien (1978). Recently, cytological aspects of encystation and penetration, the colonization of the host tissue , and the sporulation process have been investigated indepth (Kiefer *et al.* 2002, Riemann *et al*. 2002, Rumbolz *et al.* 2002, Unger *et al*. 2007). However, little is known about differences in the development of *P. viticola* within the host tissue among the susceptible European cultivars of *V. vinifera*.

Observations in the field indicate differences in the susceptibility for *P. viticola* among the European grapevine cultivars. However, up to date, only few studies comparing the susceptibility of the classical European *V. vinifera* cultivars have been undertaken. The question, if all European cultivars posses the same level of susceptibility, or if there are distinct differences among them is still topical and of great applied and scientific importance. Therefore, the aim of the present study was to characterize the level of susceptibility to *P. viticola* of common European grapevine cultivars in comparison to resistant *Vitis* genotypes. So far the level of susceptibility and resistance was characterized by visual assessment of disease incidence and severity. Now microscopical methods, described by Unger *et al.* (2007), are available to discriminate even slight differences in the infection rate, the temporal and spatial course of colonization, and the parasitation of the host tissue by *P. viticola*. In the present study the microscopical approach was combined with the visual assessment to get reliable data on susceptibility of European *V. vinifera* cultivars to *P. viticola*.

Material and Methods

Plant material and pathogen: Cuttings of 8 *V. vinifera* cultivars, common in European viticultural regions, one resistant hybrid, and three *Vitis* species (Tab. 1) were cultivated under greenhouse conditions. The European cultivars of *V. vinifera* represented susceptible genotypes, whereas the *Vitis* species were considered as more or less resistant to *P. viticola*. The hybrid *Vitis* x *vinifera* 'Solaris', resulting from multiple backcrossings of American species, *V. amurensis* and *V. vinifera* cultivars (Becker 1994, Basler 2002), are of mediate resistance. According to Unger *et al.* (2007), *V. riparia,* representing a highly resistant species, and *V. vinifera* 'Müller-Thurgau', generally considered as highly susceptible, were used as controls to assess the degree of susceptibility. A population of *Plasmopara viticola* was obtained from naturally infected plants in the vineyards around Freiburg (Germany) and maintained on *V. vinifera* 'Müller-Thurgau' grown in the greenhouse. Sporangia were raised in order to prepare an inoculum following the method of Rumbolz *et al*. (2002). For this purpose, the adaxial leaf surfaces of the plants were sprayed with a suspension of 40,000 sporangia·ml^{-1} in distilled water and covered overnight with a wet polythene bag. The inoculated plants were kept under high relative humidity (RH) (> 96 %) overnight at 24 °C. After incubation for 5 to 6 d (dpi) under ambient greenhouse conditions, the plants were again maintained under moist conditions overnight to induce sporulation. From the sporulating lesions, freshly developed sporangia were collected in centrifuge tubes using a small paintbrush. The inoculum was prepared by counting these sporangia using a Fuchs-Rosenthal chamber and adjusting to a dilution of 25,000 sporangia·ml^{-1}.

Table 1

Origin the plant material examined

Genotypes	Origin
Albariño clone 1	Centuries-old plants, MBG collection, Spain
Albariño clone 2	Centuries-old plants, MBG collection, Spain
Albariño clone 3	Centuries-old plants, MBG collection, Spain
Tempranillo	Universidad de La Rioja collection, Spain
Touriga Nacional	Estação Vitivinícola Nacional collection, Dois Portos, Portugal
Riesling	Clone FÊR 52
Müller-Thurgau	Clone FR 2
Pinot Noir	Clone FR-52-86
Pinot Blanc	Clone FR 70
Cabernet Sauvignon	Specimens Weinbauinstitut Freiburg Germany
Vitis x *vinifera* cv. Solaris	Specimens Weinbauinstitut Freiburg Germany
V. riparia	cv. Gloire de Montpellierr
V. rupestris	cv. du Lot
V. amurensis	USDA National Clonal Germplasm Repository Davis (Ca) U.S.A. DVIT1156.2

Leaf disc test: Leaf discs were prepared according to Staudt and Kassemeyer (1995) and Rumbolz *et al*. (2002). Leaves, always obtained from the same position on the shoot (5th to 6th unfolded leaf), were surface sterilized with 70 % (v/v) ethanol and subsequently rinsed in deionized water. After drying with filter paper, 12 discs with 16 mm diameter were punched out of each leaf using a cork borer, and were placed bottom side up in Bio-assay Dishes 245x245x25 mm (Nunc A/S, Roskilde Denmark) containing water agar (0.8 %).

One hundred discs of each genotype were inoculated with 50 µl of the sporangial suspension and incubated in a culture chamber for 5 d at 25 ºC and > 95 % RH under long-day conditions (white light, photosynthetic photon fluent rate (PPFR) 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ over the wave band 400 nm to 700 nm 16 h, darkness 8 h). Quantitative symptoms of infection such as disease incidence (sporulation and necrosis), disease severity (sporulation), and sporulation density were visually analyzed as independent parameters 5 d after inoculation. To score disease incidence, we determined the number of discs with sporulation per total number of discs (disease incidence of sporulation), and with necrosis per total number of discs (disease incidence of necrosis). To score disease severity, we estimated the percentage of the disc area showing symptoms of sporulation (disease severity of sporulation**)**. The sporulation density was scored subjectively after the following scheme: 0-25: lower density; 25-50: average density; 50-75: high density; 75-100: very high density. Digital photographs of the leaf discs were analysed using analySIS 3.0 software (Soft Imaging System GmbH, 1998) to measure the total surface of leaf discs and the surface of sporulation lesions and necrosis.

Detached leaf test: Twenty five whole leaves (5 leaves per 5 plant) of each genotype were placed on 0.8 % water agar (Kiefer *et al.* 2002) in the same fashion described above for the leaf discs. Leaves were inoculated with 4 or 5 50 µl drops of the sporangia suspension and incubated in a culture chamber for 5 d under the conditions described above. Observations were made 5 dpi and the infection symptoms analyzed as described above.

Plant test: All the plants examined, including the control, were grown in pots in a greenhouse (five plants per genotype) and sprayed with sporangia suspension. Incubation and sporulation proceeded as described above. After 5 to 6 dpi, symptoms of infection such as disease severity (sporulation, necrosis and oil spots), disease incidence (sporulation, necrosis and oil spots) and sporulation density (see above) were analyzed. The disease incidence was calculated as the number of leaves with sporulating lesions at the abaxial surface (disease incidence of sporulation), with necrosis (disease incidence of necrosis), or oil spots (disease incidence of oil spots**)** per total number of leaves per plant. To score disease severity we estimated the percentage of the leaf area exhibiting symptoms of sporulation (disease severity of sporulation), with necrosis (disease severity of necrosis), or oil spots (disease severity of oil spots**)**. All experiments (leaf disc test, detached leaf test and plant test) were performed in triplicate.

Microscopic analysis: For each sample time (24, 48, 72, 96, 120 hpi) and repetition of the experiment, three inoculated discs were cleared with 1M KOH, and stained with aniline blue according to Kiefer *et al.* (2002). In the stained specimens, the development of the pathogen was analyzed with an epifluorescence microscope (Axiphot Zeiss, Germany; excitation at 395-440 nm, beamsplitter FT 460 nm, longpass emission filter LP 470 nm, Plan-Neofluar objectives), equipped with a digital imaging system (AxioCam and AxioVision, Zeiss, Germany). To determine the susceptibility, the percentage of stomata successfully penetrated by *P. viticola* and the frequency of hyphae colonizing the host mesophyll were determined. For this purpose, at 24 hpi the number of stomata with a substomatal vesicle and those where a primary hyphae had already formed, as well as the total number of stomata per lesion, and at 48 hpi, the frequency of long hyphae per lesion were assessed. Additionally, the length of the hyphae was measured by means of the AxioVision (Zeiss) digital imaging system at 24 hpi and at 48 hpi, and the total number of haustoria of each hypha was quantified.

Statistical analysis: The results for each variable were examined by analysis of variance (ANOVA) to detect any significant differences. Fisher's protected test (least significant difference [LSD] method) was used to compare the level of resistance of each variety. Principal components analysis (PCA) was performed on the results of all the variables. Pearson coefficients were calculated ($P < 0.05$) to compare the data for all variables within and between methods. All calculations were performed using the GLM procedure of SAS System software, version 9.1.2. (SAS Inst, 2004).

Results

Disease incidence and severity: For disease incidence and severity significant differences were seen between the European cultivars and both the *Vitis* species and the hybrid 'Solaris' in leaf discs, detached leaves, and whole plants (Tabs 2 and 3). The inoculation experiments with leaf discs and detached leaves revealed a different susceptibility for *P. viticola* among the European cultivars (Fig. 1). When the European cultivars were compared, significant differences were seen in the leaf disc and detached leaf tests in terms of severity of sporulation and sporulation density, but no differences were found in terms of incidence of sporulation and incidence of necrosis (Tab. 2).

Comparing severity of sporulation (percentage of leaf surface covered with sporulation lesions) and sporulation density (estimated density of sporulation) in these experiments, two distinct susceptibility groups of European cultivars were distinguished: (i) 'Cabernet Sauvignon', 'Pinot Blanc', 'Pinot Noir' and 'Riesling' with a lower disease severity and sporulation density, and (ii) the three clones of 'Albariño' and 'Müller-Thurgau', 'Touriga Nacional' and 'Tempranillo', showing a higher disease severity and sporulation density (Tab. 2). Within the first group, 'Cabernet Sauvignon' expressed the lowest susceptibility, and the parameter for severity was significantly different from that of the other cultivars of this group. In both leaf discs

Table 2

Means values for each genotype in the leaf disc test and detached leaf test

Genotypes	Leaf disc test (n = 300)				Detached leaf test (n =7 5)			
	Disease incidence (%)		Disease severiy	Density sporulation (%)	Disease incidence (%)		Disease severiy	Density sporulation (%)
	sporulation	necrosis	sporulation		sporulation	necrosis	sporulation	
Albariño clone 1	100	0	48.27	100	100	0	100	100
	A[a]	A	AB	A	A	B	A	A
Albariño clone 2	100	0	51.07	100	100	0	100	100
	A	A	A	A	A	B	A	A
Albariño clone 3	100	0	27.96	66.66	100	0	75	75
	A	A	D	D	A	B	B	B
Tempranillo	100	0	48.27	75.00	100	0	100	75
	A	A	AB	C	A	B	A	B
Touriga Nacional	100	0	35.45C	100	100	0	75	100
	A	A		A	A	B	B	A
Müller-Thurgau	100	0	43.94	83.33	100	0	75	58.3
	A	A	B	B	A	B	B	C
Riesling	100	0	30.15	66.66	100	0	75	50
	A	A	CD	D	A	B	B	D
Pinot Noir	100	0	20.87EF	75	100	0	66.7	50
	A	A		C	A	B	C	D
Pinot Blanc	100	0	26.77	75	100	0	75	58.3
	A	A	DE	C	A	B	B	C
Cabernet Sauvignon	100	0	18.31	37.50	100	0	25	25
	A	A	F	E	A	B	D	E
Vitis x *vinifera* cv. Solaris	43.33	55.66	11.72	9.45	2.46	100	0	0
	B	B	G	F	B	A	E	F
Vitis rupestris	18.00	82.00	9.30	3.53	0	100	0	0
	C	C	GH	G	C	A	E	F
Vitis amurensis	9.66	90.33	4.74	0.48	0	100	0	0
	D	D	HI	H	C	A	E	F
Vitis riparia	4.66	95.33	1.29	1.18	0	100	0	0
	E	E	I	H	C	A	E	F
L.S.D (0,05)	2.87	2.80	6.04	0.71	0.71	0	0	0

Interaction genotype*sample not significant; [a]Mean separation by Fisher's protected test (least significant difference [LSD] method), at $p \leq 0.05$. Means with the same letter are not significantly different

and detached leaves, 'Tempranillo' exhibited a higher disease severity but a lower sporulation density than 'Touriga Nacional' (Tab. 2). In whole plants, differences for disease incidence and severity were also found among the European cultivars (Tab. 3). These experiments indicated the same susceptibility groups as determined with leaf discs and detached leaves. 'Cabernet Sauvignon', 'Pinot Blanc', 'Pinot Noir' and 'Riesling' were significantly different from the three clones of 'Albariño' and 'Müller-Thurgau', 'Touriga Nacional' and 'Tempranillo' in all the tested parameters, such as incidence of oil spots, sporulation, and necrosis (Tab. 3).

'Albariño' clones showed the greatest incidence of sporulation and had the largest number of oil spots. However, no significant differences were seen comparing the parameters for disease incidence and severity in whole plants of 'Albariño' with 'Tempranillo' and 'Touriga Nacional'. In the first group, 'Müller-Thurgau' showed a high incidence of sporulation (66.20 %), however had one of the lowest incidences of oil spots. Within the second group, the susceptibility of 'Riesling', 'Pinot Noir' and 'Pinot Blanc' for *P. viticola* on whole plants was nearly homogenous. As in leaf discs and detached leaves, in whole plants also 'Cabernet Sauvignon' was the least susceptible of all the European cultivars studied.

Among the *Vitis* species and the hybrid 'Solaris', significant differences in disease incidence, disease severity and sporulation density occurred in the leaf discs. Within this group, the hybrid 'Solaris' showed the highest disease incidence and severity, followd by *V. rupestris* with distinct lower values. *V. amurensis* had once more lower disease incidence and severity, and in *V. riparia,* both parameters were significantly the lowest (Tab. 2). Nearly the same order was found for the sporulation density, except that *V. amurensis* had the lowest sporulation density on leaf discs. In principle, on detached leaves and whole plants, neither oil spots nor sporulation occurred, only on the hybrid 'Solaris' a very low disease incidence was found on detached leaves (Tabs 2 and 3). On leaf discs and detached leaves of the European cultivars, no necrotic lesions were

T a b l e 3

Means values for each genotype in the plant test

Genotypes	Plant test (n = 15)						
	Disease incidence (%)			Disease severiy			Density sporulation (%)
	sporulation	necrosis	oil spots	sporulation	necrosis	oil spots	
Albariño clone 1	96.33 A	13 CD	91.66 A	100 A	5.13 E	83.40 A	91.33 A
Albariño clone 2	87.24 B	13 CD	90.00 A	75 C	8.80 D	83.33 AB	76.01 C
Albariño clone 3	72 E	12 D	63.33 C	50 E	5.50 E	75.00 D	67.91 DE
Tempranillo	83.57 BC	15 C	75 B	100 A	5.13 E	77.66 C	82.66 B
Touriga Nacional	75.55 DE	13 CD	65.00 BC	83.33 B	5.00 E	58.33 E	56.73 G
Müller-Thurgau	66.20 F	13.66 CD	20.00 G	66.66 D	1.26 FG	25.00 H	58.75 FG
Riesling	58.59 G	5 E	38.89 D	50.00 E	1.66 FG	50.00 F	63.94 EF
Pinot Noir	58.66 GH	5 E	34.66 DE	50.00 E	1.86 F	37.66 G	28.85 I
Pinot Blanc	48.16 HI	5 E	28.33 EF	46.66 EF	0.1 G	25.00 H	28.55 I
Cabernet Sauvignon	43.73 I	0.53 F	10 H	25.00 G	0.1G	15.33 I	46.03 H
Vitis x *vinifera* cv. Solaris	0.00 J	43.64 B	0.00 I	0.00 H	17.66 C	0.00 J	0 J
Vitis rupestris	0.00 J	70.40 A	0.00 I	0.00 H	30.33 B	0.00 J	0 J
Vitis amurensis	0.00 J	70.20 A	0.00 I	0.00 H	30 B	0.00 J	0 J
Vitis riparia	0.00 J	71.00 A	0.00 I	0.00 H	50.00 A	0.00 J	0 J
L.S.D (0,05)	5.79	4.18	10.40	6.50	1.67	2.41	6.15

Interaction genotype*sample not significant; [a]Mean separation by Fisher's protected test (least significant difference [LSD] method), at $p \leq 0.05$. Means with the same letter are not significantly different.

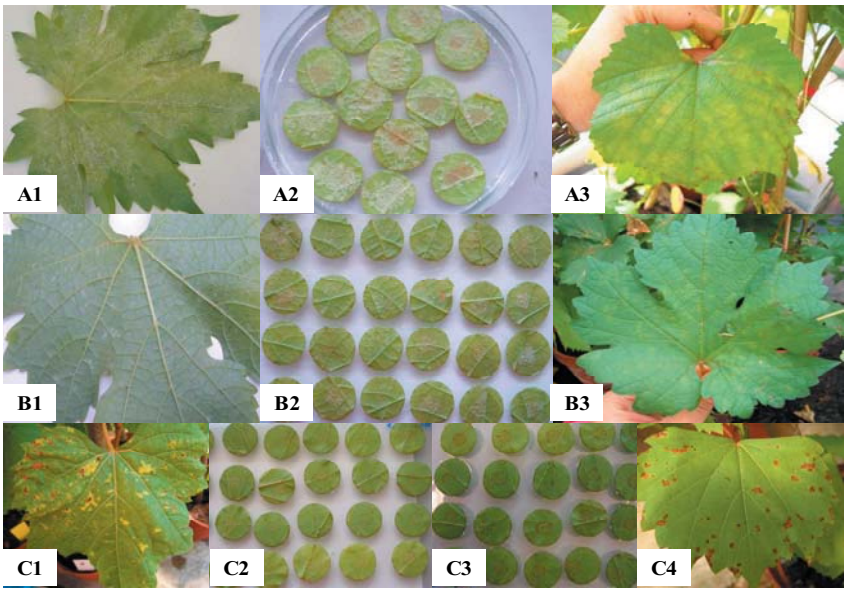

Fig. 1: Expression of symptoms of *P. viticola* on leaf discs detached leaves and plant among the susceptible and resistant *Vitis* genotypes **A1-A3**: higly susceptible varieties (cvs 'Tempranillo' and 'Albariño'); **B1-B3**: less susceptible (cv. 'Cabernet Sauvignon'), **C1-C4**: resistant (*V. riparia* and *V. rupestris*).

formed, however these occurred on the *Vitis* species and the hybrid 'Solaris'. On whole plants, the leaves of all cultivars and species tested responded with necrotic lesions after inoculation, but the incidence and severity of necrosis was significantly lower on European cultivars than on the *Vitis* species and the hybrid 'Solaris'. Among the European cultivars on whole plants, the incidence of necrosis was significantly higher on cvs 'Albariño', 'Tempranillo', 'Touriga Nacional' and 'Müller-Thurgau' than on 'Riesling', 'Pinot Noir', 'Pinot Blanc' and 'Cabernet Sauvignon'. The severity of necrosis was also significantly different, but in this case 'Müller-Thurgau' must be classified among the cultivars with lower severity (Tab. 3). The incidence and severity of necrosis on detached leaves and whole plants was significantly different among the *Vitis* species and the hybrid 'Solaris'. The highest amount of necrosis in the leaf disc test and on detached leaves occurred in *V. riparia*, whereas in the hybrid 'Solaris', incidence and severity of necrosis was significantly the lowest. The values for *V. rupestris* and *V. amurensis* ranged between the two. (Tabs 2 and 3).

High Pearson correlation coefficients ($P < 0.05$) were seen between the experiments with leaf discs, detached leaves, and whole plants with respect to all the variables studied. For example, with respect to disease severity of sporulation, a correlation coefficient of $r = 0.89$ was obtained between the leaf disc and detached leaf techniques, and of $r = 0.99$ with respect to sporulation density. The variables incidence and severity of sporulation were highly but negatively correlated ($r = -0.97$) with the variables incidence and severity of necrosis. The obtained results demonstrate that the disc test and plant tests are sufficient to study the susceptibility.

Microscopical analysis: By means of epifluorescence microscopy the mesophyll colonization by *P. viticola* in the different European *V. vinifera* cultivars, in comparison to the *Vitis* species, and the hybrid 'Solaris' was studied. This approach enabled us to compare the spatial and temporal development of the pathogen in different grapevine genotypes, and to determine even slight differences in resistance and susceptibility. As a result, all developmental steps from penetration of the stomata up to the formation of sporangiophores and sporangia were characterized. The first stages of infection occurred at 24 hpi in both the resistant and susceptible genotypes in the same period after inoculation. However, already at the beginning of the colonization of the mesophyll, distinct differences between the genotypes occurred (Fig. 2), indicating differences in resistance and susceptibility. In this first stage, the substomatal vesicle forms a primary hypha, which invades the intercellular space of the mesophyll, and the first haustorium penetrates a host cell wall (Fig. 2 (E1-E2)). In the next stage, the pathogen invades the intercellular space of the mesophyll and formes a long hypha (Fig. 2 E3-E4). At 48 hpi the hyphae branch and develop a mycelium in the susceptible genotypes.

The high percentage of stomata infected with a substomatal vesicle forming a primary hypha of 'Albariño', 'Tempranillo' and 'Touriga Nacional' indicates a high susceptibility (Tab. 4). At 24 hpi, significant differences were found for all parameters such as frequency of infected stomata per lesion, frequency of hyphae per lesion, hyphae length, and number of haustoria per hypha between the European cultivars and both, the *Vitis* species and the hybrid (Tab. 4). Among the European cultivars, 'Albariño'

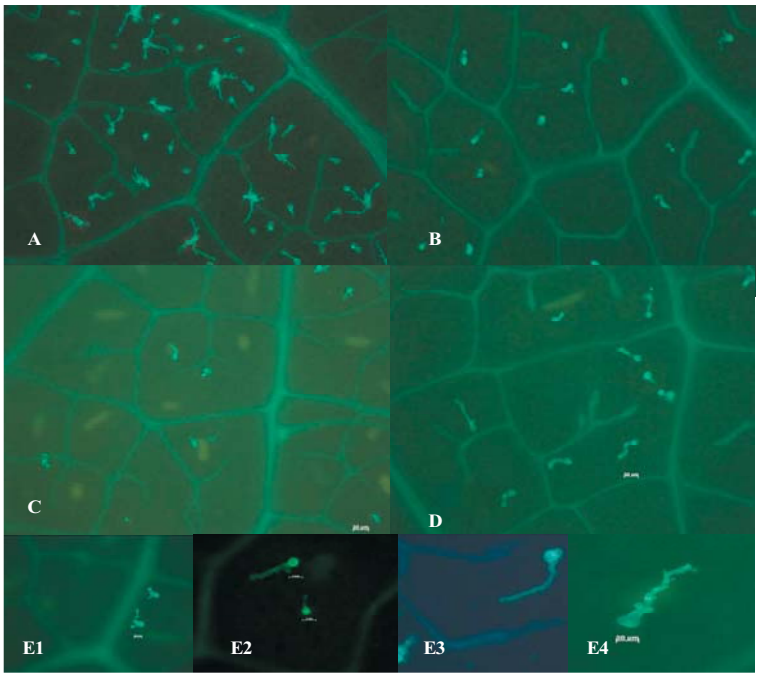

Fig. 2: Course of colonization at 24 hpi by *P. viticola* in the susceptibles European grapevine cultivars and the resistant *Vitis* species, as visualized with epifluorescence microscopy after staining with anilinblue. **A**: cv. 'Tempranillo', **B**: cv. 'Cabernet Sauvignon', **C**: *V. riparia*, **D**: *V. rupestris*, **E1-E2**: substomatal vesicle forms a primary hypha and primary haustorium (*Vitis vinifera* cv. 'Cabernet Sauvignon' and 'Tempranillo', respectively). **E3-E4**: Elongated hypha invading the intercellular space and many haustoria (*Vitis vinifera* cv. 'Albariño' and 'Tempranillo', respectively).

clones 1, 2 ,3 and 'Tempranillo', 'Touriga Nacional', and 'Riesling' showed a higher frequency of infected stomata at 24 hpi, and at this sample time in general, the development of *P. viticola* was more advanced as indicated by a higher frequency of hyphae per lesion, longer hyphae, and more haustoria per hypha. The length of hyphae varied between 60.38 µm for 'Touriga Nacional' and 155.04 µm for 'Albariño' clone 1. In contrast, in cvs 'Pinot Noir', 'Pinot Blanc', and 'Cabernet Sauvignon', the colonization of the mesophyll proceeded slower, indicated by the shorter length of hyphae and a lower frequency of haustoria per hypha (Tab. 4). However, in 'Touriga Nacional', belonging to the group of cultivars most susceptible the hyphae of *P. viticola* were smaller and the number of haustoria was lower than in the less susceptible 'Pinot Noir' (Tab. 3). At 24 hpi, a significantly higher frequency of hyphae per lesion and more haustoria were observed in 'Pinot Noir' than in 'Pinot Blanc' (Tab. 4).

At 48 hpi, no differences in the development of *P. viticola* were seen among the European cultivars. In most cultivars, hyphae grew and branched, forming a well developed mycelium which colonized the intercellular space within an intercostal field (Fig. 3). However, in contrast to the other cultivars, in 'Cabernet Sauvignon', the lateral growth and branching of hyphae were reduced and no dense mycelium was formed. In all cultivars, sporangiophores with sporangia were formed 5 to 6 d (120 hpi) after inoculation (Fig. 3).

Comparing the European cultivars with the *Vitis* species and the hybrid 'Solaris', in principle significant differences between both groups were observed for all variables such as frequency of infected stomata per lesion, frequency of hyphae per lesion, hyphae length, and number of haustoria per hypha at 24 hpi and 48 hpi. However, at 24 hpi the frequency of infected stomata per lesion in the hybrid 'Solaris' was not significantly different to 'Müller-Thurgau',

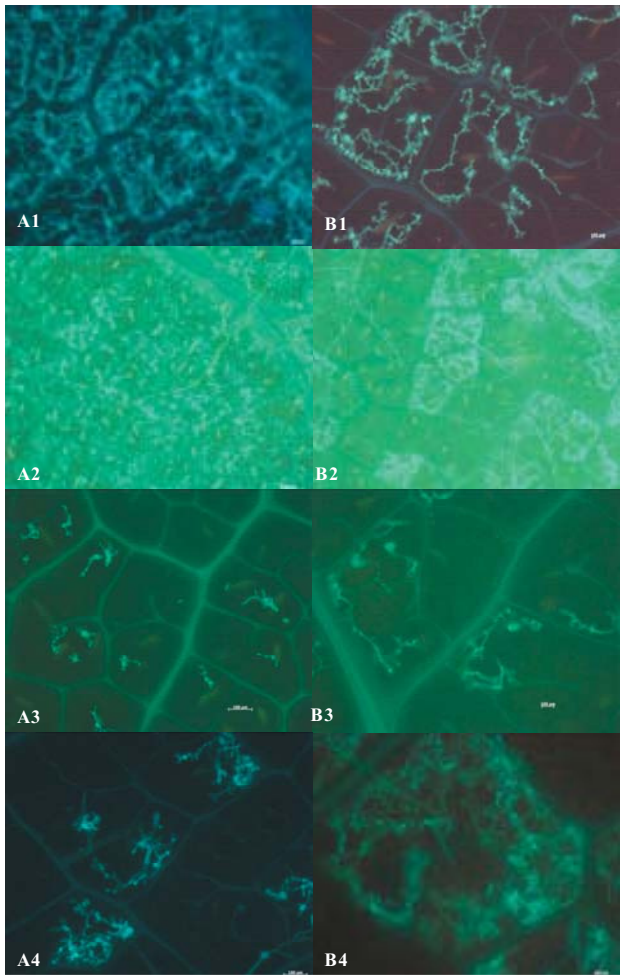

Fig. 3: Epifluorescence microscopy of the mycelium 48 hpi and 96 hpi. **A1-B1**: cvs 'Tempranillo' and 'Cabernet Sauvignon' 48 hpi; **A2-B2**: cvs 'Tempranillo' and 'Cabernet Sauvignon' 96 hpi; **A3-B3**: *V. riparia* and *V. rupestris* 48 hpi; **A4-B4**: *V. riparia* and *V. rupestris* 96 hpi.

Table 4

Means values for each genotype in microscopic analysis (24 hpi)

Genotypes	Frequency of infected stomata	Frequency hyphae	Length of hyphae (µm)	Number of haustoria
Albariño clone 1	51.29 A[a]	64.85 AB	155.04 AB	5.64 AB
Albariño clone 2	43.60 B	63.47 ABC	118.51BC	3.80 D
Albariño clone 3	30.48 D	57.41 CD	91.06 E	3.24 E
Tempranillo	42.87 C	59.42 BCD	135.42 AB	5.51 AB
Touriga Nacional	32.05 D	48.30 E	60.38 G	3.93 D
Müller-Thurgau	21.63 E	54.93 D	73.43 F	4.68 C
Riesling	28.75 D	55.21 D	88.88 E	3.88 D
Pinot Noir	22.54 E	58.02 CD	108.37 D	5.42 B
Pinot Blanc	20.50 EF	44.60 E	69.28 FG	4.97 C
Cabernet Sauvignon	20.06 EF	29.46 F	64.39 FG	2.48 F
Vitis x *vinifera* cv. Solaris	17.92 EF	9.26 G	10.13 I	0.48 GH
Vitis rupestris	12.74 G	9.16 G	41.31 H	0.77 G
Vitis amurensis	17.78 F	1.53 H	4.74 I	0.20 H
Vitis riparia	12.05 G	7.66 G	46.74 H	0.71 G
L.S.D (0.05)	4.73	6.09	10.89	0.43

Interaction genotype*sample not significant; [a]Mean separation by Fisher's protected test (least significant difference [LSD] method), at $p \leq 0.05$. Means with the same letter are not significantly different.

'Pinot Noir', 'Pinot Blanc' and 'Cabernet Sauvignon', and the same parameter was also not significantly different between *V. amurensis* and 'Pinot Blanc' and 'Cabernet Sauvignon' (Tab. 4).

At 24 hpi, in *V. rupestris* and *V. riparia,* the frequency of infection by *P. viticola*, indicated by the percentage of stomata with a substomatal vesicle and a primary hypha, was significantly lower than in the European cultivars. The first step of mesophyll colonization, the formation of a long hypha invading the intercellular space, occurred with a lower frequency, and length of this hyphae, as well as the the number of haustoria per hyphae, were reduced in all *Vitis* species and the hybrid Solaris, when compared with the European cultivars (Tab. 4). At this time, in *V. amurensis* the development of *P. viticola* was most inhibited. Two days after inoculation, the frequency of penetrated stomata in all *Vitis* species and the hybrid 'Solaris' was approximately the same as at 24 hpi (Tab. 5). Within the next 24 hpi, however, in the hybrid 'Solaris' the frequency of long hyphae increased compared to the *Vitis* species. More long hyphae with a greater length and a higher number of haustoria were observed (Tab. 5). Among the *Vitis* species, the fewest number of haustoria per hypha were counted for *V. riparia.* This difference was significant.

Principal components analysis (PCA): PCA analysis of all the recorded variables was performed to determine the level of resistance and susceptibility to downy mildew of the evaluated grapevine species and cultivars. The first two axes accounted for 93.71 % of the total variability (Prin1: 85.18 %, Prin2: 8.53 %; Fig. 4 shows the distribution of the varieties in terms of these axes). With respect to Prin1, the most important variables were: incidence of sporulation in the plant test, sporulation density and severity of sporulation in the plant test, leaf disc and detached leaf tests, frequency of hyphae per lesion, the number of haustoria, the length of the hyphae, and the incidence of necrosis in the detached leaf test (a negative correlation was recorded between the latter variable and all others). With respect to Prin2 the most important variables were: incidence of necrosis on plants, discs, and detached leaves, incidence of sporulation on leaf discs and detached leaves (a negative correlation was recorded between the latter variable and all others), incidence and disease severity of oil spots in the plant test, and frequency of infections per stoma.

The varieties assembled into four clearly distinguishable groups. With respect to Prin1, all the susceptible cultivars showed sporulation, a very low percentage of

Table 5

Means values for each resistant genotype in Microscopic Analysis (48 hpi)

Genotypes	Infection/stomata[1]	Hyphae/infection[2]	Length of hyphae (µm)	Number of haustoria
Vitis x *vinifera* cv. Solaris	21.53 A[a]	28.62 A	261.18 A	34.15 A
Vitis rupestris	10.78 B	14.00 B	238.05 A	14.43 C
Vitis amurensis	13.81 B	18.49 B	184.10 B	17.55 B
Vitis riparia	10.01 B	12.57 B	179.41 B	6.57 D
L.S.D (0,05)	3.86	6.98	51.95	2.88

Interaction genotype*sample not significant; [a]Mean separation by Fisher's protected test (least significant difference [LSD] method), at $p \leq 0.05$. [1]: Frequency of infected stomata per lesion; [2]: frequency hyphae per lesion. Means with the same letter are not significantly different.

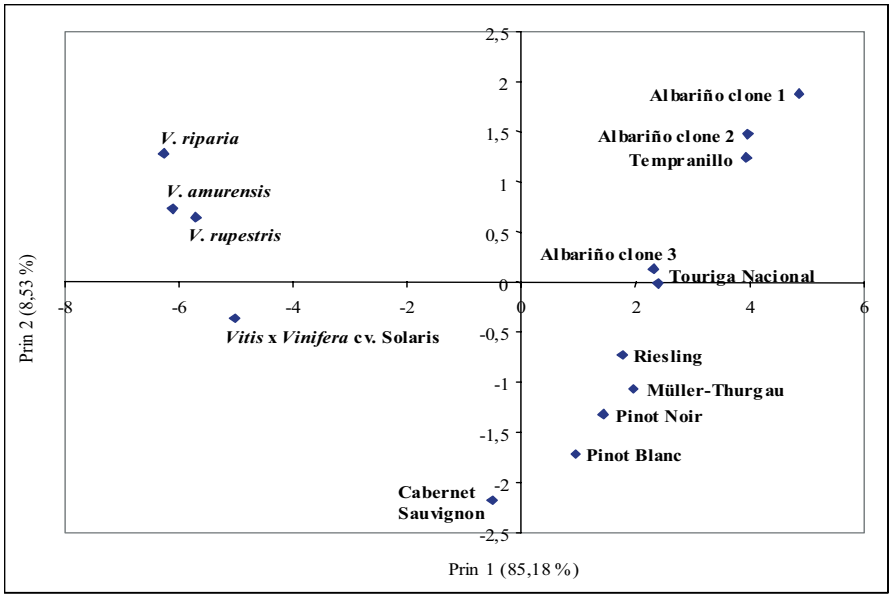

Fig. 4: Principal Components Analysis (PCA) of all the recorded variables was performed to determine the level of resistance and susceptibility to downy mildew of the evaluated grapevine species and cultivars.

necrosis and the advanced development of the *P. viticola* (large number of hyphae and haustoria and a large number of infections and hyphae per stoma), and grouped at the right of the graph. The resistant varieties, in which no or little sporulation was seen, all grouped towards the left of the graph. With respect to Prin2, the resistant varieties *V. riparia*, *V. amurensis,* and *V. rupestris*, with high levels of necrosis and little sporulation, grouped in the upper part of the graph. 'Tempranillo' and the 'Albariño' clones 1 and 2, with high disease severity in terms of incidence of sporulation and incidence of oil spots, and low disease severity in terms of incidence of necrosis, grouped at the extreme right of the graph. 'Solaris', with the greatest incidence and severity of sporulation and the lowest incidence and severity of necrosis of all the resistant varieties, was found at the bottom left of the graph. Finally, 'Touriga Nacional', 'Albariño' clone 3, 'Riesling', 'Müller-Thurgau', 'Pinot Noir', 'Pinot Blanc' and 'Cabernet Sauvignon', with a low percentage of necrosis and high incidence and severity of sporulation, as well as the most advanced development of *P. viticola*, were grouped at the lower right of the graph. Within this group, three subgroups were distinguishable, composed of 1) 'Albariño' clone 3 plus 'Touriga Nacional', 2) 'Riesling', 'Müller-Thurgau', 'Pinot Noir' and 'Pinot Blanc', and finally 3) 'Cabernet Sauvignon'.

Discussion

The statistical analysis of our inoculation experiments reveal a different level of susceptibility to *P. viticola* among the *Vitis vinifera* cultivars. As described in some previous papers (Staudt and Kassemeyer 1995, Spring 2001, Gindro *et al*. 2003) *Vitis* species and the hybrid cv. 'Solaris' express a distinct resistance against *P. viticola.* In contrast, all the European cultivars of *V. vinifera* were considered susceptible for *P. viticola* (Meluc 1981, Li 1985). Our inoculation experiments confirmed these findings and showed that *Vitis* species from North America and Asia, as well as the hybrid, descending from multiple backcrossings with European cultivars from *V. vinifera*, comprise a group of genotypes expressing resistance to *P. viticola*. Withing this group, we found significant differences for resistance parameters between the *Vitis* species we tested. We found a range from the highly resistant *V. riparia* to the hybrid 'Solaris' with a less pronounced resistance. The resistance of the species *V. rupestris* and *V. amurensis* was intermediate. Compared to the *Vitis* species and the hybrid, the group of European *V. vinifera* cultivars was susceptible for *P. viticola*. Regarding the level of susceptibility among this group, some cultivars were significantly more susceptible than others. According to the inoculation experiments, 'Riesling', 'Pinot Noir', 'Pinot Blanc', and 'Cabernet Sauvignon' can be considered as little and 'Müller-Thurgau' as intermediately susceptible, whereas 'Albariño', 'Touriga Nacional' and 'Tempranillo' are highly susceptible to *P. viticola*. Within the group of little susceptible cultivars, 'Cabernet Sauvignon' diverged showing a very low susceptibility.

Besides the assessment of incidence and severity of symptoms and sporulation, the microscopic analysis of the course of infection and colonization of the host tissue according to Unger *et al.* (2007) is a suitable method to quantify resistance and susceptibility of grapevine genotypes to *P. viticola*. In our experiments, we observed the same stages of infection and colonization, and the same time course for 'Müller-Thurgau' and *V. rupestris* as described by Unger *et al*. (2007). This microscopic analysis confirms the findings of the inoculation experiments concerning the high susceptibility of 'Albariño', 'Tempranillo' and 'Touriga Nacional', and the lower susceptibility of the other European cultivars, in particular 'Cabernet Sauvignon' with a very low susceptibility. In comparison to the European cultivars, the course of colonization of the hybrid 'Solaris' by *P. viticola* did not differ from that cultivars with low susceptibility. However, length of hyphae and number of haustoria of *P. viticola* colonizing this hybrid, were considerably different from those of the susceptible cultivars. These findings support the assumption that resistance of the hybrid 'Solaris' can be attributed to reduced development of hyphae and haustoria. In the *Vitis* species, both the delayed course of colonization and the reduction of hyphae length and number of haustoria may be caused by a defense response (Langcake *et al.* 1980, Barlass *et al*. 1987, Kortekamp *et al.* 1998).

As already described (Boso *et al*. 2006), 'Albariño' clone 1 and 'Albariño' clone 2 were the most susceptible of the European cultivars examined, whereas 'Albariño' clone 3 was less susceptible to *P. viticola*. This clone specific difference may be due to an infection by grapevine leafroll associated virus 3 (GLRaV3) of 'Albariño' clone 3, that was detected by means of ELISA (Bohnert and Boso unpubl.). An effect of virus infection on the development and sporulation of some phytopathogenic fungi has been described before (Montalbini and Umesh-Kumar 1994, Xie and Kuc 1997, Jeun and Buchenauer 2001, Musetti *et al.* 2002, 2005).

The necrosis which were formed in *Vitis* species and the hybrid 'Solaris' as response to infection with *P. viticola*, indicates a successful defense reaction as has been described for some resistant genotypes of grapevine species (Langcake *et al.* 1980, Barlass *et al*. 1987, Dai *et al.* 1995, Busam *et al.* 1997 a, b, Kortekamp *et al.* 1998, Brown *et al*. 1999 a, Kortekamp and Zyprian 2003, Unger *et al.* 2007) and may be attributed to a hypersensitivity reaction (HR) causing programmed cell death (PCD) around the infection site. The expression of peroxidase and presence of reactive oxygen species (ROS) in grapevine found by Kortekamp *et al.* (1998) as a reaction to infections with by *P. viticola* suggests, that HR is relevant for the resistant response in grapevine. This assumption is supported by the findings of Hückelhoven and Kogel (1998), Trujillo *et al*. (2004) who described the role of HR and PCD in the defense reaction of plants. The high frequency of necrotic lesions that we found on the highly resistant *V. riparia* and the low frequency of lesions on susceptible genotypes is further evidence that HR plays an important role in resistance of grapevine to *P. viticola*. Probably, HR affects the further development of *P. viticola* in a very early stage, before the development of haustoria. However, in the *Vitis* species and the hybrid 'Solaris', some long hyphae with haustoria

developed but the reduced hyphae length and number of haustoria in comparison to the European cultivars indicate additional resistance mechanisms that inhibit pathogen development at a later stage. This is in accordance with the findings of Busam *et al.* (1997), who determined the expression of pathogenesis related proteins in *Vitis* species 12 h after an attack by *P. viticola* to be a putative resistance response. Especially in the hybrid 'Solaris' Gindro *et al*. (2003) found an apposition of callose around the infection site.

In general, resistance of plants can be described as a quantitative trait ranging from highly to slightly resistant (Young, 1996). Recent findings on quantitative trait locus in the progeny of test crosses revealed that resistance in grapevine genotypes may be also quantitative (Fischer *et al*. 2004). In comparison to resistance which is precisely defined (Kranz 2003), susceptibility can be defined as lacking or delayed resistance response of the host plant. In consequence, in susceptible host genotypes the pathogen successfully penetrates the host, forms parasitation structures such as haustoria, colonizes the host tissue and completes its infection cycle. The aim of our work was to demonstrate, that in grapevine also susceptibility is quantitative and that among the European cultivars, which are regarded as susceptible, susceptibility can have different levels. The Principal Components Analysis (PCA) revealed three clusters; (i) the highly susceptible 'Albariño' clones 1 and 2, and 'Tempranillo'; (ii) the little susceptible 'Cabernet Sauvignon', 'Pinot Blanc', 'Pinot Noir', 'Müller-Thurgau' and 'Riesling'; and (iii) the resistant *Vitis* species and the hybrid 'Solaris'. This clustering and the ranging within the clusters of the European cultivars confirm that there is a continuum from highly to little susceptible. It is obvious that the little susceptibility of 'Pinot Blanc' and particularly of 'Cabernet Sauvignon' for *P. viticola* can be attributed to a low resistance. However, this assumption has to be further proven in quantitative analyses on the resistance response in the grapevine genotypes that we have studied.

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